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Identification of and Hydrogen Peroxide Production by Fecal and Vaginal Lactobacilli Isolated from Japanese Women and Newborn Infants

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We identified *Lactobacillus* isolates from Japanese women and newborn infants by a DNA-DNA hybridization method. The predominating lactobacilli were *Lactobacillus crispatus* and *Lactobacillus gasseri* in the women's vaginas and the newborns' intestines and *L. gasseri* and *Lactobacillus fermentum* in the women's intestines. All *L. crispatus* strains were exclusively strong H_2O_2 producers.

The human intestinal tract and vagina harbor a number of microorganisms which form complex and finely balanced ecosystems with their environments. Among these microbes, *Lactobacillus* spp. are believed to play an important role in stabilization of the microflora by providing an important microbial defense against vaginal and intestinal colonization by exogenous pathogenic microorganisms. Classification studies have resulted in recent taxonomic changes of human-related lactobacilli; members of the *Lactobacillus acidophilus* group have been divided into six species—*L. acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii* (7, 11)—and *Lactobacillus vaginalis* has been recently classified (4). Few studies adapted to the recent classification of *Lactobacillus* have been performed for investigation of fecal and vaginal lactobacilli (1, 2).

Although previous reports have suggested that production of H_2O_2 by lactobacilli may represent an important nonspecific antimicrobial defense mechanism in the vaginal ecosystem (5, 9, 10, 14, 16), the H_2O_2-producing lactobacilli have been seldom identified to the species level.

In this study, using a DNA-DNA hybridization method, we identified to the species level *Lactobacillus* strains which had been isolated from stool specimens of mothers and infants and vaginal swabs of women to understand the precise ecology of intestinal and vaginal lactobacilli, and we investigated their abilities to produce H_2O_2.

Reference strains used were 26 *Lactobacillus* species or subspecies (Table 1). Eighty-five fecal lactobacilli were isolates from 49 healthy mothers and 36 infants born by normal vaginal delivery at Gifu University Hospital in Gifu between 1995 and 1996; 91 vaginal lactobacilli from healthy women were isolated from 27 of the mothers mentioned above and from 64 pregnant women who visited a prenatal clinic in Gifu (16); and 6 vaginal lactobacilli from women with bacterial vaginosis (BV) were isolates from 6 pregnant women (16). MRS agar (Becton Dickinson and Company, Cockeysville, Md.) was used for anaerobic culture of isolates. Lactobacilli were identified as catalase-negative, non-spore-forming, microaerophilic gram-positive rods producing abundant lactate as an end product with or without small amounts of acetate (12).

Bacterial DNA was extracted by the method described previously (13). DNA was labeled with PHOTOPROBE biotin (Vector Laboratories Inc., Burlingame, Calif.), according to the manufacturer's instructions. DNA-DNA hybridization was

TABLE 1. Reference strains of *Lactobacills* species used for DNA hybridization studies

Group and species	Strain
L. delbrueckii group (obligately homofermentative)[a]	
L. acidophilus	JCM 1132
L. amylophilus	JCM 1125
L. amylovorus	JCM 1126
L. crispatus	JCM 1185
L. delbrueckii subsp. *bulgaricus*	JCM 1002
L. delbrueckii subsp. *delbrueckii*	JCM 1012
L. delbrueckii subsp. *lactis*	JCM 1248
L. gallinarum	JCM 2011
L. gasseri	JCM 1025
L. helveticus	JCM 1120
L. jensenii	JCM 1146
L. johnsonii	JCM 2012
L. delbrueckii group (facultatively heterofermentative)[a]	
L. acetotolerans	JCM 3825
L. casei-Pediococcus group (obligately homofermentative)[a]	
L. salivarius subsp. *salicinius*	JCM 1150
L. salivarius subsp. *salivarius*	JCM 1231
L. casei-Pediococcus group (facultatively heterofermentative)[a]	
L. casei	JCM 1134
L. paracasei subsp. *paracasei*	JCM 1181
L. paracasei subsp. *tolerans*	JCM 1171
L. plantarum	JCM 1149
L. rhamnosus	JCM 1136
L. casei-Pediococcus group (obligately heterofermentative)[a]	
L. brevis subsp. *brevis*	JCM 1059
L. buchneri	JCM 1115
L. fermentum	JCM 1173
L. fructivorans	JCM 1117
L. reuteri	JCM 1112
L. vaginalis	JCM 9505

[a] Data from the review by Vandamme et al. (17).

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TABLE 2. *Lactobacillus* species isolated from stool and vaginal specimens of Japanese women and newborn infants

Species	No. of specimens (%)			
	Stool		Vagina	
	Mothers	Infants	Mothers and women without BV	Women with BV
L. crispatus	4 (8.2)	6 (16.7)	48 (52.7)	2 (33.3)
L. fermentum	9 (18.4)	2 (5.6)	5 (5.5)	1 (16.7)
L. gasseri	11 (22.4)	12 (33.3)	19 (20.8)	2 (33.3)
L. paracasei subsp. *paracasei*	5 (10.2)	3 (8.3)	0	0
L. paracasei subsp. *tolerans*	3 (6.1)	2 (5.6)	0	0
L. plantarum	4 (8.2)	0	3 (3.3)	0
L. reuteri	2 (4.1)	0	0	0
L. rhamnosus	2 (4.1)	1 (2.8)	0	0
L. salivarius subsp. *salicinius*	3 (6.1)	3 (8.3)	1 (1.1)	0
L. salivarius subsp. *salivarius*	3 (6.1)	6 (16.7)	1 (1.1)	0
L. vaginalis	0	0	8 (8.8)	1 (16.7)
Unidentified	3 (6.1)	1 (2.8)	6 (6.6)	0
Total	49	36	91	6

carried out as described elsewhere (6) with modifications. Genomic DNA was adjusted to 100 μg/ml with distilled water. DNA diluted 10-fold with phosphate-buffered saline (pH 7.4) containing 0.1 M $MgCl_2$ was distributed into a microtiter plate (100 μl/well). Hybridization of DNA from reference strains with denatured, biotinylated sample DNA was carried out for 4 to 5 h at 45°C.

H_2O_2 production by *Lactobacillus* strains was tested with MRS agar supplemented with 0.25 mg of tetramethylbenzidine (Sigma, St. Louis, Mo.) per ml and 0.01 mg of horseradish peroxidase (Sigma) per ml (5). Inoculated plates were anaerobically incubated for 2 days at 37°C. H_2O_2 production was ranked as strongly positive, weakly positive, or negative according to the intensity of blue color development.

Ten species and subspecies from mothers' stools and eight species from stools of infants were detected, whereas three strains from mothers and one from an infant remained unidentified (Table 2). *L. gasseri* was the most commonly found species in both mothers and infants, but the second most predominant species differed between the mothers and infants studied: *Lactobacillus fermentum* in the mothers and *L. crispatus* and *Lactobacillus salivarius* subsp. *salivarius* in the infants.

The predominant species found in the vaginas of 91 healthy women included *L. crispatus*, *L. gasseri*, and *L. vaginalis* (Table 2). In women with BV, *L. crispatus* and *L. gasseri* were common.

A total of 172 strains identified by DNA hybridization were tested for H_2O_2 production (Table 3). All *L. crispatus* strains were strongly positive for H_2O_2 production, and all *L. gasseri* and *L. vaginalis* strains were strongly or weakly positive, while *L. paracasei* and *L. plantarum* strains were all negative. Of six isolates from women with BV, two were *L. crispatus*, two were *L. gasseri*, one was *L. vaginalis*, and one was *L. fermentum*; all but the *L. fermentum* strain were positive for H_2O_2 production.

We found that *L. crispatus* was the predominant vaginal lactobacillus, followed by *L. gasseri*, in Japanese women. Giorgi et al. (8), who used DNA homology techniques, reported that *L. crispatus* and *L. jensenii* were the predominating lactobacilli in healthy women. These data suggest that *L. crispatus* is a common vaginal lactobacillus in healthy women in both Japan and Western countries.

The present study showed that the predominant species in stools were *L. gasseri*, *L. fermentum*, and *L. paracasei* subsp. *paracasei* for women and *L. gasseri*, *L. crispatus*, and *L. salivarius* subsp. *salivarius* for infants. Benno et al. (2) reported that *L. gasseri* was the dominant species among lactobacilli isolated from the intestinal tracts of elderly Japanese people. Meanwhile, in the studies in Western countries, the *L. acidophilus* group, *L. salivarius*, and *L. fermentum* were usually recovered from stools of adults and infants (3), and the largest taxa in the rectal mucosa of healthy adults were *L. plantarum*, *L. rhamnosus*, and *L. paracasei* subsp. *paracasei* (1). These results indicate that the intestinal lactobacilli may be different between Japanese and Western people. Although the reasons for this dissimilarity are unclear, it may be speculated that the inhabiting *Lactobacillus* species in stools and in the intestinal mucosa are different and that differences between Japanese and Western diets may influence the *Lactobacillus* species resident in the intestine.

We found that all *L. crispatus* strains were strong H_2O_2 producers, while *L. paracasei* and *L. plantarum* strains were negative for H_2O_2 production, and that there was relatedness between *Lactobacillus* species and H_2O_2 production regardless of whether or not the isolates were from women with BV. Nagy

TABLE 3. H_2O_2 production by lactobacilli isolated from stool and vaginal specimens

Species	No. of strains tested	H_2O_2 production	No. of isolates			
			Stool		Vagina	Total (%)
			Mother	Infant		
L. crispatus	60	Strongly positive	4	6	50	60 (100)
		Weakly positive	0	0	0	0 (0)
		Negative	0	0	0	0 (0)
L. gasseri	44	Strongly positive	6	6	6	18 (40.9)
		Weakly positive	5	6	15	26 (59.1)
		Negative	0	0	0	0 (0)
L. fermentum	17	Strongly positive	4	0	1	5 (29.4)
		Weakly positive	2	1	1	4 (23.5)
		Negative	3	1	4	8 (47.1)
L. paracasei subsp. *paracasei*	8	Strongly positive	0	0	0	0 (0)
		Weakly positive	0	0	0	0 (0)
		Negative	5	3	0	8 (100)
L. paracasei subsp. *tolerans*	5	Strongly positive	0	0	0	0 (0)
		Weakly positive	0	0	0	0 (0)
		Negative	3	2	0	5 (100)
L. plantarum	7	Strongly positive	0	0	0	0 (0)
		Weakly positive	0	0	0	0 (0)
		Negative	4	0	3	7 (100)
L. reuteri	2	Strongly positive	1	0	0	1 (50)
		Weakly positive	0	0	0	0 (0)
		Negative	1	0	0	1 (50)
L. rhamnosus	3	Strongly positive	0	0	0	0 (0)
		Weakly positive	1	1	0	2 (66.7)
		Negative	1	0	0	1 (33.3)
L. salivarius subsp. *salicinius*	7	Strongly positive	1	0	0	1 (14.3)
		Weakly positive	1	2	0	3 (42.9)
		Negative	1	1	1	3 (42.9)
L. salivarius subsp. *salivarius*	10	Strongly positive	0	3	0	3 (30)
		Weakly positive	0	3	1	4 (40)
		Negative	3	0	0	3 (30)
L. vaginalis	9	Strongly positive	0	0	4	4 (44.4)
		Weakly positive	0	0	5	5 (55.6)
		Negative	0	0	0	0 (0)

et al. (15), who identified lactobacilli by phenotypic characteristics, showed that the ability to produce H_2O_2 was more likely to be associated with the origins of strains (BV or non-BV) than with the *Lactobacillus* species themselves. Since the methods used for detection of the ability to produce H_2O_2 were almost the same between our study and that of Nagy et al., differences in lactobacillus identification might be responsible for the contradictory results.

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